REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
22-10-2008	Final Report	12-Jun-2006 - 31-Aug-2008

4. TITLE AND SUBTITLE
Stabilization of Proteins by Polymer Conjugation via ATRP

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W911NF-06-C-0068

5c. PROGRAM ELEMENT NUMBER
206023

6. AUTHORS
Lance Mabus, Jason Berberich, Bhalchandra Lele, Virginia Depp

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Agentase LLC
2240 William Pitt Way

Pittsburgh, PA 15238 -1358

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
50332-CH-CDP.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
US Army Research Office has funded this 2-year basic research effort by ICx-Agentase to study new techniques of modifying native enzymes with polymers via atom transfer radical polymerization to increase their solubility and utility in organic solvents and to increase their stability in body. Protein-initiated ATRP would enable us to overcome many problems in conventional technology that modifies proteins with polyethylene glycol or “pegylation”. These problems include lack of control on number of polymer chains attached per protein, low yield, tedious purifications, and less-than-optimal stabilization. The first goal of this study is to develop new technique/s to modify enzymes with hydrophobic polymers and dissolve high concentrations of

15. SUBJECT TERMS
ATRP, Solvent solubilization, therapeutic proteins, hydrophilic polymers, protein stabilization

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Jason Berberich
U	U	U	SAR		19b. TELEPHONE NUMBER 412-423-2131

Report Title

Stabilization of Proteins by Polymer Conjugation via ATRP

ABSTRACT

US Army Research Office has funded this 2-year basic research effort by ICx-Agentase to study new techniques of modifying native enzymes with polymers via atom transfer radical polymerization to increase their solubility and utility in organic solvents and to increase their stability in body. Protein-initiated ATRP would enable us to overcome many problems in conventional technology that modifies proteins with polyethylene glycol or "pegylation". These problems include lack of control on number of polymer chains attached per protein, low yield, tedious purifications, and less-than-optimal stabilization. The first goal of this study is to develop new technique/s to modify enzymes with hydrophobic polymers and dissolve high concentrations of modified enzymes in organic solvent and apply them in self decontaminating paints and coatings. The second goal of the study was to study the potential of using of ATRP modified proteins for therapeutic applications in vivo. Chymotrypsin and BChE conjugates synthesized by ATRP were demonstrated and were highly active and much more stable in mouse serum as compared to conventionally pegylated enzymes.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

V. Depp, J.L. Kaar, A.J. Russell, B.S. Lele. Enzyme Sheathing Enables Nanoscale Solubilization of Biocatalyst and Dramatically Increases Activity in Organic Solvents. Biomacromolecules 9, 1348 (2008).

Number of Papers published in peer-reviewed journals: 1.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Protein-initiated ATRP: Superior alternative to pegylation for stabilizing biologics. Protein Engineering Summit 2008 Boston MA.

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Native protein-initiated ATRP: A viable and potentially superior alternative to pegylation for stabilizing biologics. Submitted to Acta Biomaterialia.

Number of Manuscripts: 1.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Final Report

1. **Date:** August 31, 2008

2. **Reporting Period:** June12, 2006-August 31, 2008

3. **Proposal Number:** 50332-CH-CDP

4. **Agreement Number:** W911NF-06-C-0068

5. **Contract Title: Stabilization of Proteins by Polymer Conjugation via ATRP**

6. **Authors:** Jason Berberich, Lance Mabus, Virginia Depp, Bhalchandra Lele

7. **Classification:** Unclassified

8. **Project Objective:** US Army Research Office has funded this 2-year basic research effort by ICx-Agentase to study new techniques of modifying native enzymes with polymers via atom transfer radical polymerization to increase their solubility and utility in organic solvents and to increase their stability in body. Protein-initiated ATRP would enable us to overcome many problems in conventional technology that modifies proteins with polyethylene glycol or "pegylation". These problems include lack of control on number of polymer chains attached per protein, low yield, tedious purifications, and less-than-optimal stabilization. The first goal of this study is to develop new technique/s to modify enzymes with hydrophobic polymers and dissolve high concentrations of modified enzymes in organic solvent and apply them in self decontaminating paints and coatings. The second goal of the study was to study the potential of using of ATRP modified proteins for therapeutic applications *in vivo*. Chymotrypsin and BChE conjugates synthesized by ATRP were demonstrated and were highly active and much more stable in mouse serum as compared to conventionally pegylated enzymes.

9. **Objectives of the work:**

    - Develop polymerization method for hydrophobic monomers using model enzymes as initiators of atom transfer radical polymerization
    - Identify alternative technique/s for solvent solubilization of enzymes
    - Demonstrate control on the number of ATRP initiators conjugated to proteins
    - Demonstrate control on the molecular weight of polymer grown from protein surface
    - Conduct stability study on protein polymer conjugates and compare with conventional pegylation

10. Results and Discussion:

Solvent soluble decontaminating enzymes for paint coating

ATRP of methacrylate monomers using model protein chymotrypsin

Proposed research is based upon our previous publication that described synthesis of uniform protein-polymer conjugates by ATRP. Chymotrypsin was modified with 2-bromoisobutyramide moieties which are most commonly used as initiators in ATRP. Number of initiators conjugated to chymotrypsin was controlled by controlling the ratio of [protein]: [2-bromoisobutyryl bromide] in protein "activation" reaction. These protein-initiators efficiently conducted polymerization of vinyl monomers in aqueous medium to synthesize highly active enzyme-polymer conjugates. In this effort, we proposed to test our hypothesis that protein activated with ATRP initiators can initiate polymerization of hydrophobic monomers in organic solvent to synthesize solvent soluble protein-polymer conjugates. The reaction scheme proposed to test this hypothesis comprised initial dissolution of chymotrypsin-initiator in organic solvent by forming its ion pair with surfactants followed by ATRP of methacrylate monomers in organic solvent, and removal of surfactants with washing in hexane.

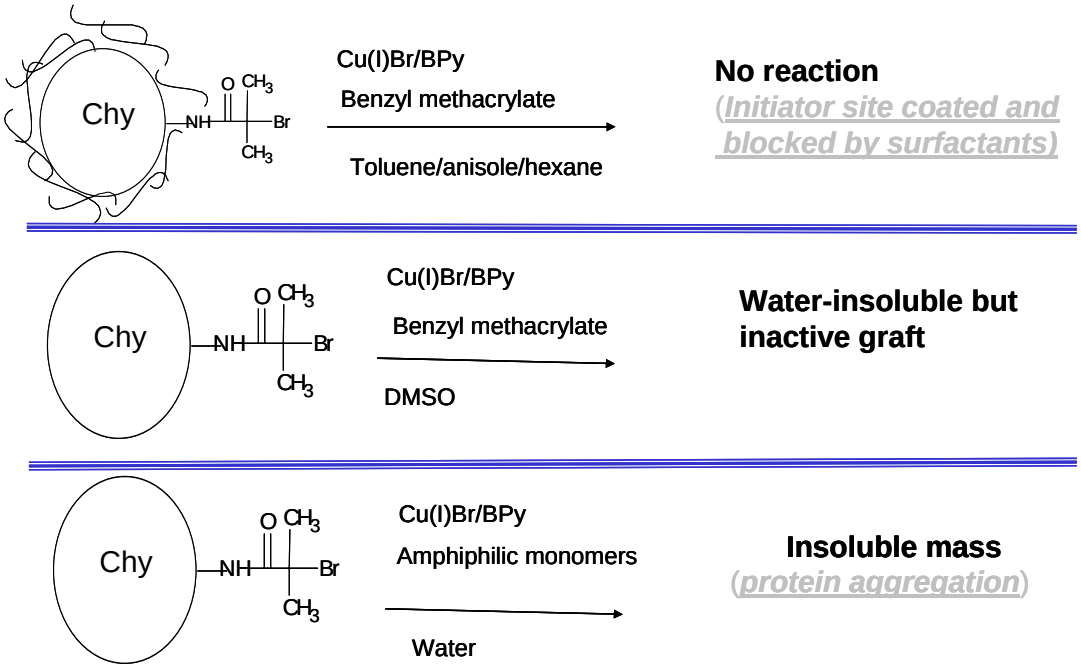


Figure 1: Schematic summarization of results obtained for various initial experiments performed before arriving at an optimized solution

It was found that ion paired enzyme-initiator did not initiate ATRP. This was the case for different solvents, polymerization conditions, and ion paired chymotrypsin initiators used. Benzyl methacrylate is known to undergo facile ATRP under ambient temperatures to give well-defined poly(benzyl methacrylate). Failure of AOT: chymotrypsin-initiator to initiate polymerization indicated that this could be due to inaccessibility of 2-bromoisobutyramide sites to initiation catalysts and/or monomers due to coating of surfactant around protein surface. To confirm this, we conducted ATRP of benzyl methacrylate using chymotrypsin-initiator alone as the protein-initiator dissolved in dimethylsulfoxide, a highly polar organic solvent which dissolves proteins up to 1-5 mg/mL conc. but also denatures them.

Our studies demonstrated that ion pairing of chymotrypsin-initiator with AOT hindered polymerization process and that chymotrypsin-initiator alone was able to initiate ATRP of hydrophobic monomers in organic solvent to produce solvent soluble but water insoluble enzyme-hydrophobic polymer conjugate. However, practical applicability of this finding is limited due to the use of strongly polar organic solvent which tend to denature enzymes. To avoid the use of aggressive polar solvent, we decided to use water as the medium for polymerization of amphiphilic monomers which have solubility in both water and organic solvents. When growing polymer chain attains certain length, we expected precipitation of enzyme-polymer conjugate from water. These experiments demonstrated that there is a need of spacer between the protein surface and initiator site to prevent protein aggregate formation.

ATRP of amphiphilic methacrylate monomers using chymotrypsin modified with a PEG spacer between protein surface and initiator

Figure 2 illustrates the strategy we followed to alleviate protein aggregation during polymerization of amphiphilic monomers initiated by proteins. Since pegylation of a surface is known to resist protein adsorption we envisaged placing a spacer of polyethylene glycol between chymotrypsin and ATRP initiator would help in preventing protein aggregation during formation of a hydrophobic polymer chain that will be attached to chymotrypsin through a long, “nonfouling” PEG chain.

Our studies showed that protein aggregation during polymerization of hydrophobic monomers initiated directly from protein surfaces can be alleviated by placing a spacer of PEG between protein and ATRP initiator. However, this compromises solvent solubility of the resulting enzyme-PEG-block copolymer. These conjugates have solvent solubility that is similar to pegylated enzymes.

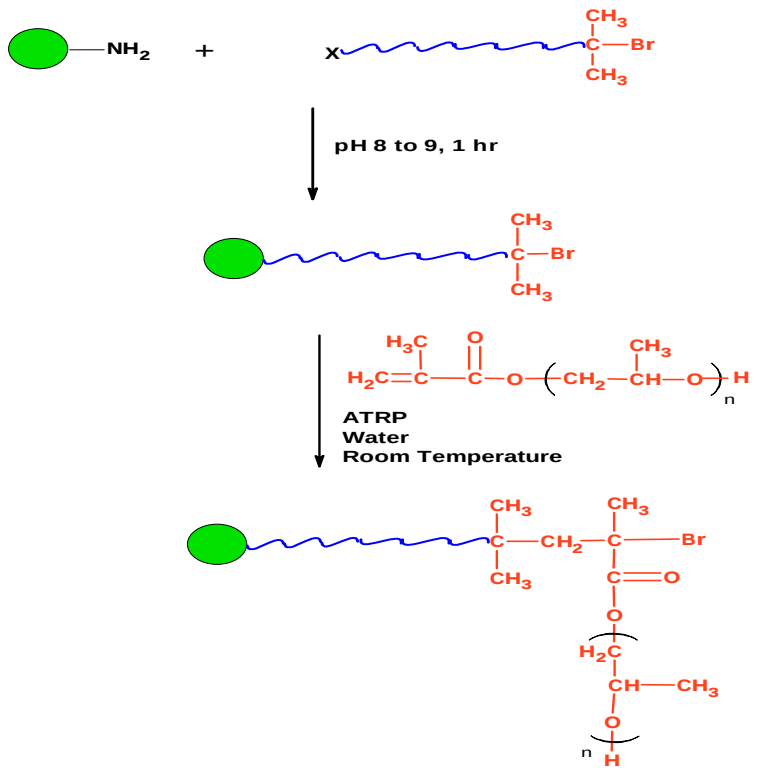


Figure 2: Schematic representation of PEG spacer strategy to overcome protein aggregation during polymerization of hydrophobic monomers.

Alternative technique of enzyme sheathing with amphiphilic copolymer for solvent solubilization

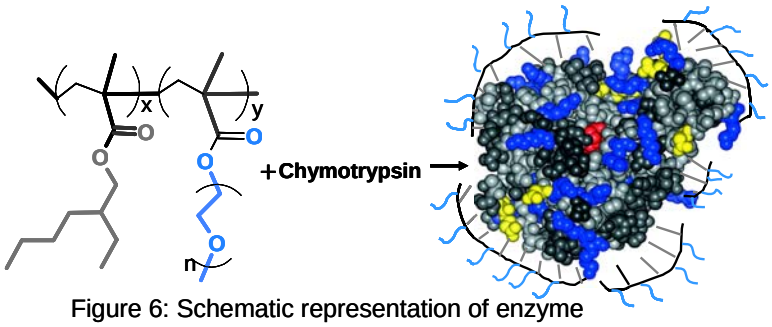


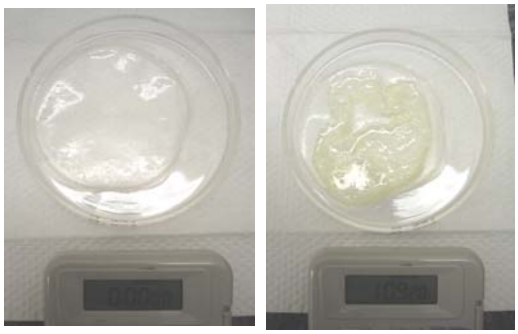
Figure 6: Schematic representation of enzyme sheathing in amphiphilic comb shaped copolymer

Synthesizing block copolymers from protein surfaces is an elegant approach to increase the solubility of enzymes in organic solvents but it is difficult to practice it in large scale and be useful for bulk applications like paints and coatings. Therefore we decided to apply the knowledge that we gained from the previous tasks to develop a simple, scalable method to increase solvent solubility of modified enzymes. It was observed that presence of both hydrophobic and hydrophilic monomers is needed in protein initiated ATRP to prevent protein aggregation and obtain soluble conjugate. This prompted us to design and synthesize a random copolymer of hydrophilic and hydrophobic monomers and complex

it with enzymes. Amphiphilic copolymers are known to interact with proteins. We envisioned coating or sheathing of our designed random copolymer on enzymes for increasing their solvent solubility. We synthesized a random copolymer of 30% w/w 2-ethylhexyl methacrylate and 70% w/w monomethoxy poly(ethylene glycol)-methacrylate (MPEG-methacrylate) to test in this new technique using chymotrypsin (CT) and horseradish peroxidase (HRP) as model enzymes. Schematic representation of sheathing enzymes in amphiphilic copolymer is shown in Figure 6.

Application of enzyme sheathing technique to synthesize poly(methyl methcarylate) films containing organophosphate hydrolase

Organophosphorous hydrolase (OPH) that was sheathed in poly(2-ethylhexyl methacrylate-co-MPEG-methacrylate) was soluble in solvents such as toluene, dichloromethane etc. To test the effectiveness of this technique we dissolved polymer sheathed OPH (100 mg) in 20 mL dichloromethane solution of poly(methyl methacrylate) (250 mg). The solution was allowed evaporate slowly in a Teflon mold to obtain a transparent film containing uniformly distributed OPH. As shown in Figure 11, OPH incorporated in polymer film was active against paraoxon, a simulant of V series nerve agents.



0 min after adding paraoxon

30 min after adding paraoxon

Figure 11: OPH (10 mg) containing poly(methyl methacrylate) film active against paraoxon.

Summary

We have developed two new techniques for enhancing solvent solubility of enzymes. The first technique comprises growing hydrophobic polymer chains from surface of enzymes modified with initiators of atom transfer radical polymerization. Presence of hydrophilic PEG spacer was found to be necessary between protein and the initiator site to prevent insoluble aggregate formation during polymerization of hydrophobic monomers from the surface of protein. Enzyme-block copolymer conjugate obtained after polymerization retains catalytic activity and is soluble in solvents such as toluene, chloroform etc.

The second technique utilizes hydrophobic interactions between proteins and amphiphilic copolymers to sheath the enzymes such as chymotrypsin and horseradish peroxidase in matrix of amphiphilic random copolymer of hydrophilic and hydrophobic monomers.

Sheathed enzymes exhibit solubility in toluene to up to 5 mg protein per mL, which is five fold higher than that reported for enzymes pegylated or modified in other traditional ways. Particle size of sheathed enzymes in toluene is 5-10 nm which is much smaller than that reported for pegylated enzymes. Sheathed chymotrypsin and peroxidase exhibited 6-36 fold higher activity than that of respective pegylated enzyme in toluene. These finding were applied to organophosphorous hydrolase. The enzyme was sheathed in copolymer matrix and dissolved in dichloromethane solution of poly(methyl methacrylate). Clear films containing uniformly distributed enzyme exhibited activity against paraoxon.

Stabilization of Proteins by Polymer Conjugation via ATRP

Demonstrate control on the number of ATRP initiators conjugated to proteins

One of the problems in conventional pegylation is lack of control on number of polymer chains that are conjugated to a protein. In this task we demonstrated that it is possible to control the average number of ATRP initiators conjugated to native proteins and thereby limit the maximum number of polymer chains that can attach to a protein. We also demonstrated that complete modification of protein with ATRP initiators is possible. Thus a protein-initiator, free of native proteins can be used to initiate ATRP of vinyl monomers and obtain protein-polymer conjugate that is free of native protein, which is difficult to get rid off without tedious column chromatography.

Many enzymes were reacted with 2-bromoisobutyryl bromide or sulfo-NHS ester of 2-bromoisobutyric acid by varying the molar ratio of protein to the acylating reagent. Protein-initiators synthesized in this way were characterized by MALDI-TOF spectrometry. Figures 5 shows MALDI-TOF spectra of chymotrypsin modified with various initiators.

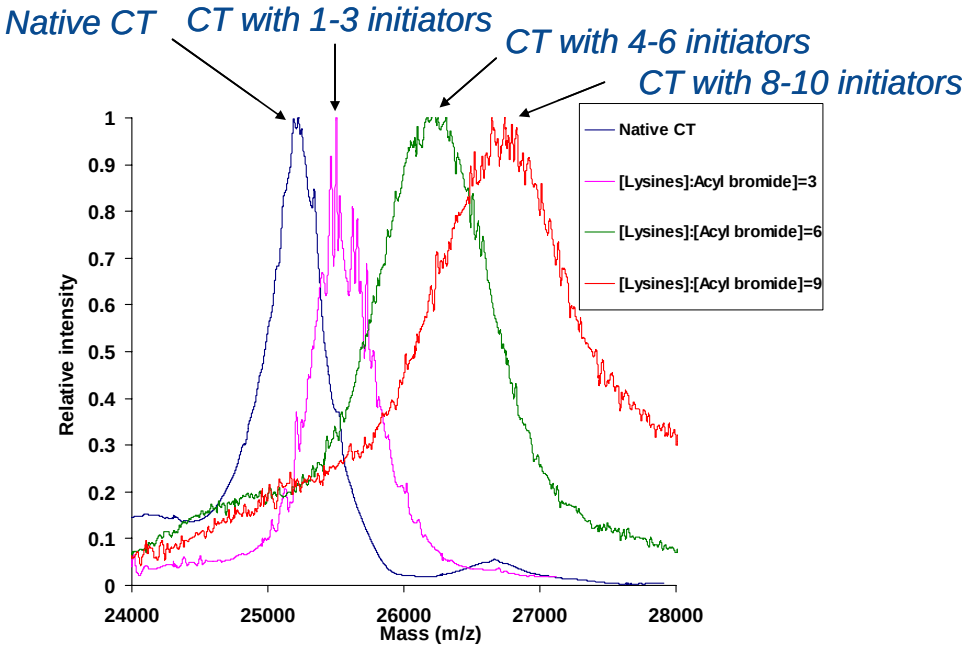


Figure 5: MALDI-TOF spectra of chymotrypsin-initiators

All protein-initiators retained enzymatic activity after modification with ATRP initiators. Enzymes modified with 1-3 initiators retained >80% activity. As the number of conjugated initiators increased further, residual activity retention decreased.

Demonstrate control on the molecular weight of polymer grown from protein surface

One of the unique advantages of protein-initiated ATRP is that it allows controlling the molecular weight (MW) of the polymer chain growing from the surface of the protein. Thus it allows synthesizing low to very high MW polymer conjugates which are otherwise difficult to synthesize by conventional pegylation. We modified model enzyme chymotrypsin with 1-3 initiators of ATRP and conducted ATRP of monomer 2-methacryloyloxyethyl phosphorylcholine (MPC) by varying the monomer to initiator molar ratio to obtain polymer-protein conjugates of low to high MW. Conjugates obtained were characterized by gel permeation chromatography using polyethylene oxide standards. Due to very high polymerizability of MPC monomer all conjugates obtained were high MW and out of range of standards used. We then scaled down the monomer to initiator ratio and synthesized conjugates of poly(MPC), poly(N-2-hydroxypropyl methacrylamide) (poly(HPMA)), and poly(monomethoxy-polyethylene glycol-methacrylate) (poly(MPEGMA) with chymotrypsin via ATRP. These polymer-protein conjugates were characterized by advanced size exclusion chromatography coupled with detectors for light scattering, viscometer, and refractive index. Table I shows that ATRP allows synthesis of 50-450 kDa MW polymer-proteins conjugates which is difficult via conventional conjugation reactions due to steric limitations.

Table I: Molecular weight characterization data for PPGs

PPG	Mw (Da)[1]	Mn (Da)[1]	Mw/Mn[1]	Rh[1] (nm)
CT	28,100	26,300	1.06	2.2
CT-graft-poly(HPMA)	56,500	40,200	1.40	3.5
CT-graft-poly(MPEGMA)	146,800	116,400	1.26	6.4
CT-graft-poly(MPC)	497,500	283,500	1.75	13

[1]Determined by size exclusion chromatography coupled with a tetra detector analyzer.
Mw = Weight average molecular weight, Mn = Number average molecular weight, Rh = Hydrodynamic radius.

Conduct stability study on protein-polymer conjugates and compare with conventional pegylation

We modified various enzymes such as trypsin, subtilisin, horseradish peroxidase, lysozyme, asparaginase, organophosphorous hydrolase etc., by ATRP and by conventional pegylation. In vitro mouse and human serum stability study was studied for native, pegylated and ATRP-modified enzymes. It was observed that many off-the-shelf enzymes were very stable and a meaningful data could no be obtained. However, lysozyme and asparaginase showed instability in mouse and human serum and provided meaningful results. We observed that conjugates synthesized by ATRP were more stable than the corresponding pegylated conjugates. Stability study results for lysozyme is shown in Figure 6.

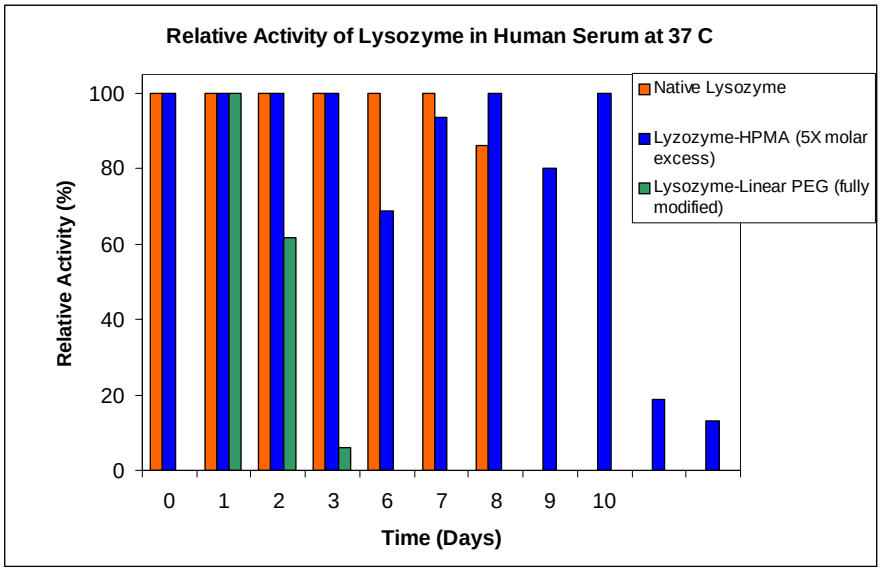


Figure 6: In vitro human serum stability study of lysozyme (protein conc. 1mg/mL)

Transition ATRP technology to butyrylcholinesterase (BChE) stabilization

We applied the technology developed to stabilization of BChE which is used as nerve agent scavenging enzyme to mitigate human exposure to nerve agents. BChE from equine serum was modified by ATRP and pegylation and in vitro serum stability was compared. As shown in Figure 7, we observed that conjugates synthesized by ATRP were marginally more stable than pegylated BChE. We note that recombinant human BChE is far more unstable than BChE from equine serum and therefore should give better results for stabilization by ATRP against pegylation, which is known to produce conjugate with short circulation life.

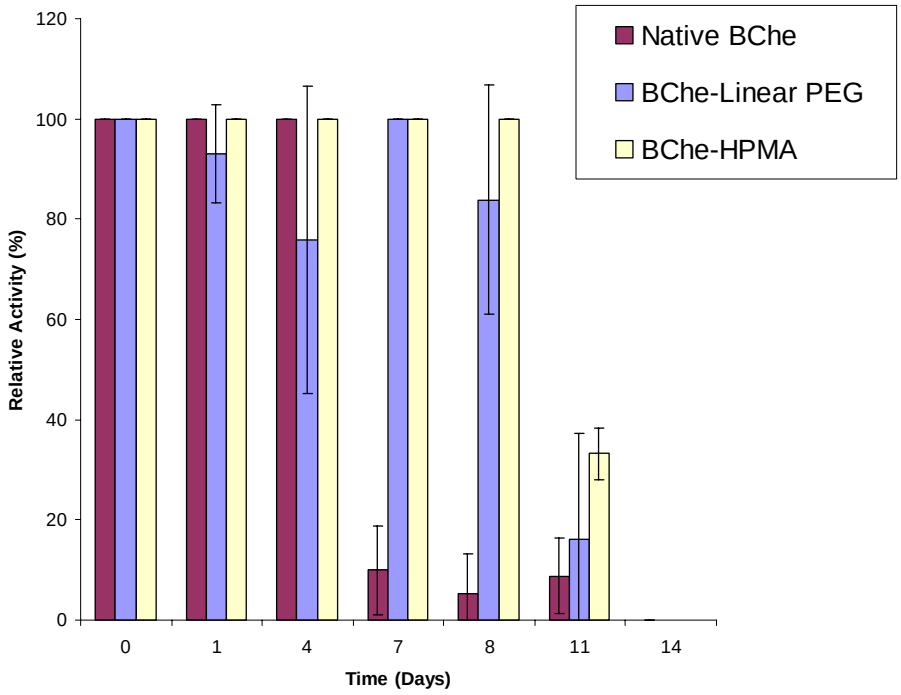


Figure 7: In vitro human serum stability study for BChE (protein conc. 0.1 mg/mL)

Summary

Protein-initiated ATRP technology was developed further to demonstrate its potential for synthesizing polymer-BChE conjugates exhibiting greater serum stability than pegylated BChE. Results point to viability of applying ATRP to human recombinant BChE and conduct further work in vitro and in vivo.

Number of advantages of protein-initiated ATRP were found over conventional pegylation that are summarized in Table III

Table III: Advantages of protein-initiated ATRP over pegylation

Conventional Pegylation of Native Proteins	Native protein-initiated ATRP
Lack of control on number of PEG chains conjugated per protein	Controls average number of ATRP initiators conjugated per protein and therefore limits maximum number of polymer chains that can be grown from protein
Limited range molecular weights of PEGs that are capable of forming conjugates efficiently	Ability to synthesize very high molecular weight polymers from proteins
Presence of unmodified protein in conjugate	Conjugate is free of unmodified protein
Tedious, low yield purification	Simple, high yield purification
Drastic drop in activity of conjugate	Retention of high activity in conjugate
In vitro serum stability lesser than ATRP	In vitro serum stability greater than pegylation

11. Presentations: Protein-initiated ATRP: Superior alternative to pegylation for stabilizing biologics. Protein Engineering Summit 2008 Boston MA.

12. **Papers submitted:**

Native protein-initiated ATRP: A viable and potentially superior alternative to pegylation for stabilizing biologics. Submitted to *Acta Biomaterialia.*

V. Depp, J.L. Kaar, A.J. Russell, B.S. Lele. Enzyme Sheathing Enables Nanoscale Solubilization of Biocatalyst and Dramatically Increases Activity in Organic Solvents. *Biomacromolecules* 9, 1348 (2008).